Original Article

Molecular Prevalence of Human Papillomavirus Types 16 and 18 in Oral Squamous Cell Carcinoma using Real-time PCR

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ABSTRACT

Human papillomavirus (HPV) has been established as a causative agent in the development of oral squamous cell carcinoma (OSCC). Specifically, HPV types 16 and 18 are known to be prevalent in oral cancers. This cross-sectional study aimed to determine the prevalence of HPV types 16 and 18 in OSCC cases in Qazvin province, Iran. Thirty-eight paraffin-embedded samples of OSCC were selected, and DNA extraction was performed using the Roche High Pure FFPE DNA isolation kit. The quality of the extracted DNA was assessed through PCR amplification of the human β-Globin gene. The HPV detection was carried out using SYBR green-based real-time PCR with GP5+ and GP6+ primers targeting the L1 region of HPV. The HPV genotyping was conducted on positive samples using specific primers. Statistical analysis was performed between HPV infection in OSCC and age, sex, and anatomical location. This study analyzed 38 biopsy specimens obtained from male and female OSCC patients, with an average age of 64 years. Among these samples, 13 tested positive for HPV, resulting in a prevalence rate of 34.2%. The age group with the highest HPV infection rate was 61-70 (10.5%) years. Notably, HPV type 16 was detected in 21.0% of samples, HPV type 18 in 10.5%, and other viral subtypes in 2.6%. No statistically significant correlation was found between HPV prevalence and gender or age. The findings indicated that 34.2% of OSCC samples in the Qazvin province harbor HPV, with types 16 and 18 being the most common in tumors affecting the tongue. Additionally, no association was observed between HPV infection and age or gender. To address HPV as a risk factor for OSCC, public health initiatives such as vaccination, awareness campaigns, and accessible healthcare services should be implemented. They are, furthermore, incorporating HPV DNA testing into practice.

Keywords: Human Papillomavirus, Iran, Oral squamous cell carcinoma, Real-Time PCR

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1. Introduction

Oral squamous cell carcinoma (OSCC) is a malign disease worldwide with a dramatic effect on health and life quality, with more than 377,700 cases worldwide in 2020 (1). The OSCC is known as the most common type of oral cancer, with high morbidity and mortality in patients with head and neck cancers (2). Various risk factors have been connected to oral cancer; among them, alcohol consumption and tobacco smoking are the most typical connected risk factors of OSCC in 74% of cases with OSCC (3). The Human papillomaviruses **(**HPVs) are a group of various and prevalent DNA viruses that infect epithelial cells of the mucosa and skin. The HPV lesions are usually clinically imperceptible, but enduring infection with high-risk HPV types resulted in malignancies at diverse anatomic locations in the neck, head, and anogenital region (4). The HPV16 and HPV18, as major high-risk types, are closely connected to malignant tumors (5). Although the association between OSCC and HPV was first proposed in 1983, the existence of HPV DNA was approved two years after, using in situ hybridization. The interesting point is that HPV types 16 and 18 contribute to more than 70% of cases of all HPV-related cancers (6). The HPV types 16 and 18 raise tumorigenesis via expression of oncoproteins, including the E6 and E7, which inactivate the tumor suppressors P53 and RB1, respectively (7). The HPV role in the growth of head and neck cancers is not obvious, mainly because its prevalence is not closely 100% as in cervical carcinoma. The HPV incidence is variant in head and neck cancers (8). In Iran, the prevalence of HPV in oral cancers ranges from 9.0% in Shiraz to 50% in Rasht. These differences in HPV prevalence are related to the intraoral location, geographical area, type of lesion, and grading (9). Initial studies indicate that the prevalence of HPV-positive oral cancers is increasing in Iran (10). Despite the presence of considerable preventative policies, HPV-related cancer remains one of the causes of mortality in different regions of the world, especially in developing countries. There is no denying that increasing knowledge about the prevalence of HPV is necessary to put a better plan into practice for vaccination and treatment. Therefore, in this study, we estimate the prevalence of HPV types 16 and 18 in OSCC in the Qazvin province, Iran.

2. Materials and Methods

2.1. Study area and study population

This study was carried out in Qazvin, located in north-central Iran, in a wide fertile plain at the southern foot of the Elburz Mountains with a population of 1,284,000.

2.2. Sample collection

In this cross-sectional study, 38 archived formalin-fixed and paraffin-embedded OSCC biopsy specimens were tested at the Oral Pathology Laboratory located in the Faculty of Dentistry of Qazvin University of Medical Sciences as a province referral laboratory. These specimens were received from 20 (52.6%) women and 18 (47.4%) men with ages ranging from 31-95 years (mean age of 64±17 years).

2.3. Extraction of DNA

Genomic DNA was isolated using a purification kit (Roche High Pure FFPE DNA isolation kit, Germany). Before DNA extraction, deparaffinization was perfirmed by the xylene-ethanol deparaffinization method (11). Following, all extracted DNA was stored at -80°C until the time of molecular tests.

2.4. Quality control of DNA Extraction

The DNA quality was assessed by PCR using primers PC04 (5′-CAACTTCATCCACGTTCACC-3′) and GH20 (5′-GAAGAGCCAAGGACAGGTAC-3′) that amplify a 268 bp product from the human *β-Globin* gene (12).

2.5. Molecular identification of HPV

Positive samples of β-Globin were subjected to HPV detection using SYBR green-based real-time PCR by primers GP5+: 5'-TTTGTTACTGTGGTAGATACTAC-3' and GP6+: 5' AAAAATAAACTGTAAATCATATTC-3' that amplifies a 150 bp product by targeting the L1 region of HPV (13). Following real-time PCR amplification, each reaction was performed in a final volume of 20 µL, containing 10 µL SYBR green PCR Master mix (TAKARA BIO INC Japan), 0.5 µL 10 pM of each forward and reverse primer, 2 ml of DNA template, 6.6 µL PCR grade water, and 0.4 ml ROX Reference Dye. Initial denaturation was at 95°C for 2 min, followed by 40 cycles, each cycle including denaturation at 94°C for 30 sec, annealing at 40°C for 2 min, and extension at 72°C for 90 sec.

2.6. HPV genotype identification

The SYBR green-based real-time PCR assay was performed using two specific primers (HPV16F: 5'-TGCTAGTGCTTATGCAGCA-3', HPV16R: 5'-TTACTGCAACATTGGTACATGG-3' and HPV18F: 5'-CGCCACGTCTAATGTTTCTG-3', HPV18R: 5'-CCTGTGATAAAGGACGCGA-3') on all positive tested samples in the previous section for the detection of HPV types 16 and 18. A final reaction volume consisted of 20 µL containing 10 µL SYBR green PCR Master mix (TAKARA BIO INC Japan), 0.5 µL 10 pM of each forward and reverse HPV16F and HPV18 primers and 2 ml of DNA template, 6.6 µL PCR grade water and 0.4 ml ROX Reference Dye. DNA was amplified by real-time PCR (Applied Biosystems, CA, USA) with SYBR green dye for detection, and each cycle consists of 2 min at 95°C, 40 cycles of 10 sec at 95°C, 30 sec at 60°C and 72°C for 30 sec.

2.7. Statistical Analysis

The association between HPV infection in OSCC and age, sex, and anatomic location was analyzed using Fisher's exact tests and Chi-square with the SPSS software (version 16). A *P*-value less than 0.05 was considered statistically significant.

3. Results

3.1. General

The study group included 38 archived formalin-fixed and paraffin-embedded OSCC biopsy specimens tested at the School of Dentistry Qazvin University of Medical Sciences. Out of 38 patients with OSCC, 18 (47.4%) were men and 20 (52.6%) were women, with a mean age of 64 ± 17, and a distribution range of 31-95, classified into seven groups (Fig 1). The OSCC lesions' locations were in the jaw bone 6/38 (15.8%), buccal mucosa 7/38 (18.4%), oral floor 3/38 (7.9%), tongue 16/38 (42.1%), lip 3/38 (7.9%), and other areas 3/38 (7.9%).

3.2. Prevalence of HPV types 16 and 18 using real-time PCR

From a total of 38 formalin-fixed and paraffin-embedded OSCC after molecular test, 13/38 (34.2%) cases were HPV positive. The prevalence of HPV among women and men was estimated 40.0% (8/20) and 27.8 % (5/18), respectively. The age group of 61-70 (10.5%) years had the highest HPV infection rate. Although HPV infection was prevalent in female compared to male patients, the difference was not statistically significant (40.0% vs. 27.8%, P=0.428). Our findings also showed non-significant associations between age and HPV infection (P=0.714). The HPV16 and HPV18 were detected in 8/38 (21.0%) and 4/38 (10.5%) cases, respectively. Of them, 1/38 (2.6%) was related to other viral subtypes. The overall prevalence of HPV in total in different locations, including tongue 6/38 (15.8%), jaw bone 3/38 (7.9%), buccal mucosa 3/38 (7.9%), and oral floor 1/38 (2.6%) is presented in Figure 2. It should be noted that the prevalence of HPV within the location of lesions is mentioned in Table 1. Although statistically no significant difference was shown between HPV and lesion location (P=0.536), HPV was more prevalent in tongue samples.

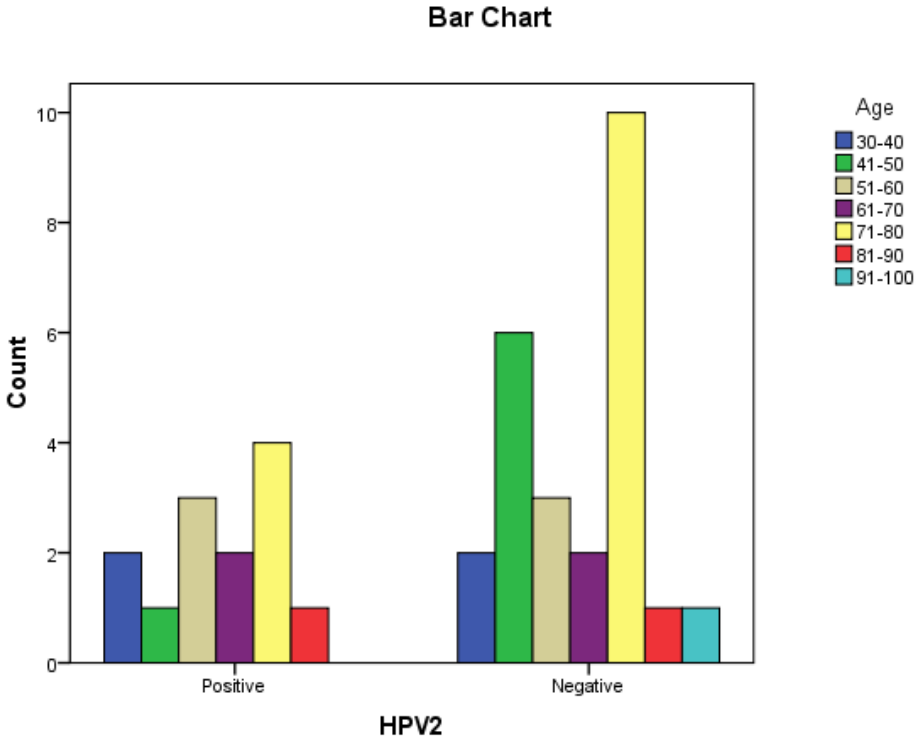

Figure 1: Prevalence of HPV among age groups.

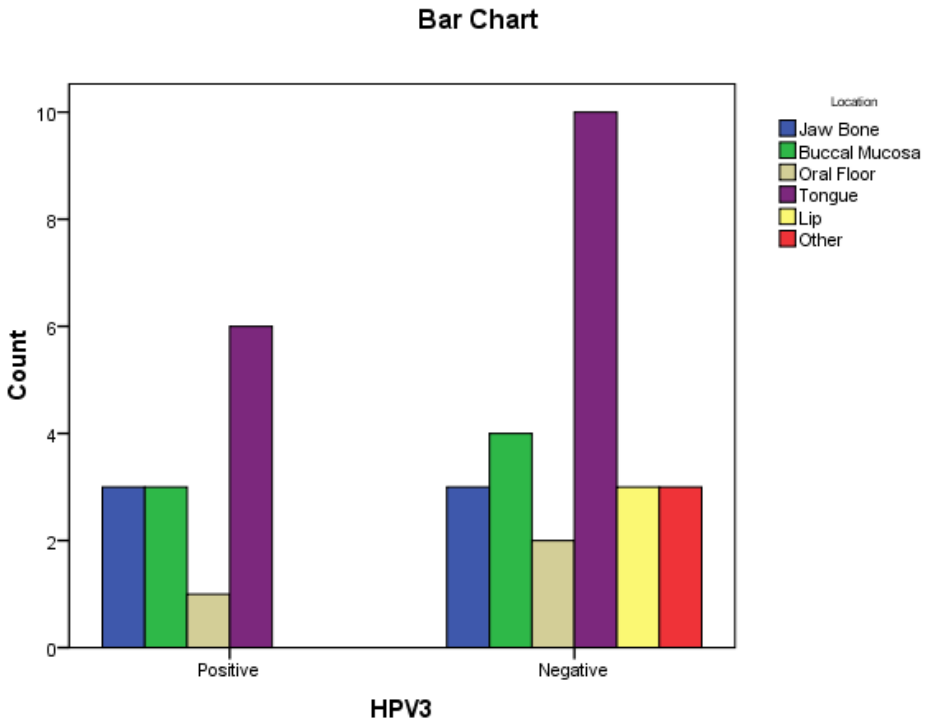

Figure 2: Prevalence of HPV within total.

Table 1: HPV prevalence within location of lesions

HPV Pos/Neg	Location					
	Jaw bone	**Buccal mucosa**	**Oral floor**	**Tongue**	**Lip**	**Other**
HPV Positive	3/6 (50.0%)	3/7 (42.9%)	1/3 (33.3%)	6/16 (37.5%)	0/3 (0.0%)	0/3 (0.0%)
HPV Negative	3/6 (50.0%)	4/7 (57.1%)	2/3 (66.7%)	10/16 (62.5%)	3/3 (100.0%)	3/3 (100.0%)

4. Discussion

Although numerous studies have confirmed the causative role of HPV infection in oral squamous cell carcinoma worldwide, its role as a risk factor in OSCC has been contentious (14). In the current study, the prevalence of HPV and its genotypes were estimated using real-time PCR in OSCC. These days, a considerable interest exists in using molecular methods in epidemiologic studies to estimate the prevalence of pathogens, including high-risk HPV types (15), which are considered the gold standard method for HPV detection in clinical specimens (16). Many studies have also shown that the prevalence of HPV in OSCC differed from 0% in Japan (17) and 64.5% in India (18). This broad spectrum variability might be described by various factors, including sample collection techniques, disease stage, sensitivity of the used methods, and demographic factors (19). There is no denying the fact that HPV is regarded as one of the risk factors of OSCC; accordingly, estimating the prevalence rate of HPV and its genotypes in OSCC seems to be necessary. The findings of this study revealed that 34.2% (13/38) of formalin-fixed and paraffin-embedded OSCCs tested were positive for

HPV. Of these 13 positive samples, 61.5% (8/13), 30.8% (4/13), and 7.6% (1/13) were found to be positive for HPV 16, HPV 18, and other HPV types, respectively. Findings of a study by Hadi Razavi Nikoo (16) showed the prevalence of HPV-linked OSCC was 36%, which is in agreement with our study. In another study conducted by Tabatabai, they reported that HPV prevalence was 43%, and they also estimated that HPV16 is the most prevalent type, which is approximately in line with our results (20). In another study conducted in 2018 in Iran on oral samples, the HPV prevalence rate was reported at 13.0%, which was less than the current study findings (21); these differences in the prevalence of HPV can be related to geographical differences (9). As well as in a parallel study to our findings conducted in Korea, the prevalence of HPV was evaluated at 36%, and the more common HPV type was HPV-16 (22). In the current study, HPV was more prevalent in women (40.0%) than men (27.8%) groups, which was similar to the study by Seifi (23). In a similar study reported by Tsimplaki, HPV infection was more prevalent in women compared to men, and the presence of HPV was not associated with age (24). The findings of this study were against the research by Ashraf and Kreimer that the prevalence of HPV in men is higher than in women (25). The prevalence of high-risk HPV highly varies in different studies between women and men in different geographic regions (26). Heterogeneity among studies about this can be also associated with the sexual behavior of patients (20). In our study, this non-significant correlation may be related to our patient's ages that majority of them were above 50 years old. Tongue is the most prevalent location in OSCC (27). In this study, HPV prevalence in OSCC was more prevalent in tongue samples similar to Ashraf et al. and Makvandi studies conducted in Iran (26, 28); in those studies, HPV was more prevalent in tongue compared to other locations of the lesions. In general, it seems that due to strong cultural changes in lifestyle and sexual behaviors, studies related to the prevalence of HPV in OSCC need to be repeated and re-evaluated. Therefore, the results of the present study would be important from this point of view. The high prevalence of HPV16 and 18 in OSCC suggests that there is a pressing need for future implementations of public health interventions, including vaccination, increasing people's awareness, sanitation, sexual health, appropriate healthcare, and ongoing screening of HPV. Furthermore, HPV DNA testing with high sensitivity and positive predictive value should become a main tool in OSCC screening.

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Authors' Contribution
M.A. and F.A. analyzed and interpreted the data. M.A. and S.A.A. were involved in the study design. H.A. and M.A. writing first draft of the manuscript. A.M. carried out the statistical analysis. S.Z. obtained resources. M.A. and H.S. revised the manuscript. All authors read and approved the final manuscript.

Ethics
The research was approved by the Qazvin University of Medical Sciences Ethics in Research Committee (Ethics code: IR.QUMS.REC.1399.355). All data and experiments were performed in accordance with Ethics in Research Committee guidelines and regulations. Additionally, the informed consent was obtained by each participant.

Conflict of Interest
The authors declare no conflict of interest.

Data Availability
The data that support the findings of this study are available on request from the corresponding author.

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The authors received no external funding for this study. The study was self-funded by the authors.

Limitation
Some limitations of this study are as follows: In this study, we studied the most common high-risk HPV genotypes (16 and 18) and other genotypes have not been assessed. Another limitation of this study goes back to our small sample size that was highly related to the willingness of patients to continue treatment in Tehran, the capital of Iran which is very nearby to Qazvin.

References

1. Li H, Zhang Y, Xu M, Yang DJJocr, oncology c. Current trends of targeted therapy for oral squamous cell carcinoma. 2022;148(9):2169-86.
2. Gharat SA, Momin MM, Bhavsar C. Oral squamous cell carcinoma: current treatment strategies and nanotechnology-based approaches for prevention and therapy. Critical Reviews™ in Therapeutic Drug Carrier Systems. 2016;33(4).

3. Chattopadhyay I, Verma M, Panda M. Role of Oral Microbiome Signatures in Diagnosis and Prognosis of Oral Cancer. Technol Cancer Res Treat. 2019;18:1533033819867354.
4. Prigge ES, von Knebel Doeberitz M, Reuschenbach M. Clinical relevance and implications of HPV-induced neoplasia in different anatomical locations. Mutat Res Rev Mutat Res. 2017;772:51-66.
5. Gan L-L, Zhang H, Guo J-H, Fan M-W. Prevalence of human papillomavirus infection in oral squamous cell carcinoma: a case-control study in Wuhan, China. Asian Pacific Journal of Cancer Prevention. 2014;15(14):5861-5.
6. Serrano B, Brotons M, Bosch FX, Bruni L. Epidemiology and burden of HPV-related disease. Best Pract Res Clin Obstet Gynaecol. 2018;47:14-26.
7. Deshpande R, Raina P, Shinde K, Mansara P, Karandikar M, Kaul-Ghanekar RJP, et al. Flax seed oil reduced tumor growth, modulated immune responses and decreased HPV E6 and E7 oncoprotein expression in a murine model of ectopic cervical cancer. 2019;143:106332.
8. Parkin DM, Bray F, Ferlay J, Pisani P. Global cancer statistics, 2002. CA Cancer J Clin. 2005;55(2):74-108.
9. Aghaeipour F, Salehiniya H, Abbaszadeh H. Prevalence of human papillomavirus (HPV) in oral mucosal lesions in Iran: A systematic review and meta-analysis. Journal of Medical Virology. 2021;93(11):6089-99.
10. Razavi SM, Siadat S, Rahbar P, Hosseini SM, Shirani AM. Trends in oral cancer rates in Isfahan, Iran during 1991-2010. Dental research journal. 2012;9(Suppl 1):S88.
11. Alinezhadi M, Makvandi M, Kaydani GA, Jazayeri SN, Charostad J, Talaeizadeh AT, et al. Detection of High-Risk Human Papillomavirus DNA in Invasive Ductal Carcinoma Specimens. Asian Pac J Cancer Prev. 2022;23(9):3201-7.
12. Ritari J, Hultman J, Fingerroos R, Tarkkanen J, Pullat J, Paulin L, et al. Detection of human papillomaviruses by polymerase chain reaction and ligation reaction on universal microarray. PLoS One. 2012;7(3):e34211.
13. Sankaranarayanan R, Budukh AM, Rajkumar R. Effective screening programmes for cervical cancer in low-and middle-income developing countries. Bulletin of the World Health Organization. 2001;79(10):954-62.
14. Tsimplaki E, Argyri E, Xesfyngi D, Daskalopoulou D, Stravopodis DJ, Panotopoulou E. Prevalence and expression of human papillomavirus in 53 patients with oral tongue squamous cell carcinoma. Anticancer Res. 2014;34(2):1021-5.
15. Wang X, Tian X, Liu F, Zhao Y, Sun M, Chen D, et al. Detection of HPV DNA in esophageal cancer specimens from different regions and ethnic groups: a descriptive study. 2010;10:1-8.
16. Nikoo HR, Ardebili A, Ravanshad M, Rezaei F, Teimoori A, Khanizadeh S, et al. E6-specific detection and typing of human papillomaviruses in oral cavity specimens from Iranian patients. Iranian Biomedical Journal. 2017;21(6):411.
17. Kabeya M, Furuta R, Kawabata K, Takahashi S, Ishikawa Y. Prevalence of human papillomavirus in mobile tongue cancer with particular reference to young patients. Cancer science. 2012;103(2):161-8.
18. Khanna R, Rao G, Tiwary S, Rai A, Khanna S, Khanna A. Detection of human papilloma virus 16 and 18 DNA sequences by southern blot hybridization in oral leukoplakia and squamous cell carcinoma. Indian Journal of Surgery. 2009;71:69-72.
19. Aghaeipour F, Salehiniya H, Abbaszadeh HJJoMV. Prevalence of human papillomavirus (HPV) in oral mucosal lesions in Iran: A systematic review and meta-analysis. 2021;93(11):6089-99.
20. Tabatabai SH, Nabieyan M, Sheikhha MH, Zarmehi S, Tadbir AA, Ahmadi NA. Detection of Human papillomavirus 16 and 18 types in oral squamous cell carcinoma patients in Yazd, Iran: A Case-Control Study. Archives of Advances in Biosciences. 2015;6(1).
21. Akhondnezhad M, Haghshenas MR, Ghasemi M, Mousavi T. The prevalence and genotyping of human papillomavirus in patients with oral tumors in health centers and clinics of Mazandaran in Iran. Virusdisease. 2018;29:297-302.
22. Lee S, Cho N, Choi E, Baek S, Kim W, Shin D, et al. Relevance of human papilloma virus (HPV) infection to carcinogenesis of oral tongue cancer. International journal of oral and maxillofacial surgery. 2010;39(7):678-83.
23. Seifi S, Kermani IA, Dolatkhah R, Kermani AA, Sakhinia E, Asgarzadeh M, et al. Prevalence of oral human papilloma virus in healthy individuals in East azerbaijan province of iran. Iranian journal of public health. 2013;42(1):79.
24. Tsimplaki E, Argyri E, Xesfyngi D, Daskalopoulou D, Stravopodis DJ, Panotopoulou E. Prevalence and expression of human papillomavirus in 53 patients with oral tongue squamous cell carcinoma. Anticancer research. 2014;34(2):1021-5.
25. Kreimer AR, Villa A, Nyitray AG, Abrahamsen M, Papenfuss M, Smith D, et al. The epidemiology of oral HPV infection among a multinational sample of healthy men. 2011;20(1):172-82.
26. Ashraf MJ, Hosseini S, Monabati A, Valibeigi B, Khademi B, Abedi E, et al. The Prevalence of Human Papilloma Virus in Squamous Cell Carcinoma of Oral Tongue. Iranian journal of pathology. 2017;12(2):144-9.
27. Tsikouras P, Bothou A, Paraskevopoulos K, Anthoulaki X, Chalkidou A, Tsirkas I, et al. HPV induced tongue cancer in pregnancy: Diagnosis and the role of vaccination in management. Clin Obstet Gynecol. 2020;6:1-7.
28. Makvandi M, Jalilian S, Faghihloo E, Khanizadeh S, Ramezani A, Bagheri S, et al. Prevalence of Human Papillomavirus and Co-Infection with Epstein-Barr Virus in Oral and Oropharyngeal Squamous Cell Carcinomas. Asian Pacific journal of cancer prevention : APJCP. 2022;23(11):3931-7.